IGF-1 (Mouse/Rat) ELISA

For the quantitative determination of IGF-1 in serum or plasma of mice and rats.

Please read carefully due to Critical Changes, e.g., plate washing instructions.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	22-IG1MS-E01
Size:	96 wells
Version:	240112 Version 5 - ALPCO January 30, 2012

ALPCO Diagnostics

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Mouse/Rat IGF-I ELISA

- is suited for IGF-I determination in serum and plasma of **mice and rats**
- high sensitive: **0.029 ng/ml** analytical sensitivity; required sample volume is very small
- is **fast**: incubation time a total of 2 hours
- Single Standards with **0.5, 2.5, 6, 12, 18 ng/ml** recombinant IGF-I are provided in the Kit
- 2 Control Sera are provided for quality control
- uses **high affinity antibodies** against m/r IGF-I
- Microtiter plates are separately breakapart

Intended Use

Measurement of IGF-I in mouse / rat serum and plasma.

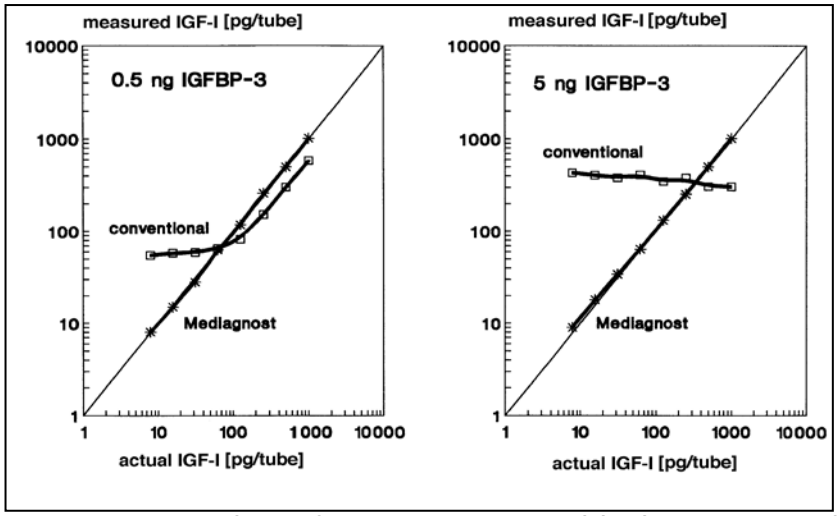
INTRODUCTION

Beside different cell culture models and studies with human patients, mice and rats are suitable model organisms for basic research and pre-clinical studies.Thus, this test system was developed as a tool for IGF-I measurements in mice and rat for usage in research and pre-clinical studies. Even if the comparability of mice and humans is limited some background information on the *human* IGF-I system follows:

Insulin-like growth factors (IGF) I and II play a pivotal role in regulating the proliferation and differentiation of many cell types (1-3). IGF-I is identical with Somatomedin C (Sm-C) (4) and has a molecular weight of 7649 daltons (5). Its major regulators are growth hormone (GH) and nutrition (6). In contrast to many other peptide hormones, IGFs are avidly bound to specific binding proteins (IGFBP). The seven IGFBPs which are known at present (7,8,22) either bind IGF-I and IGF-II with similar affinities or show a preference for IGF-II (9,10).

A major problem of IGF-I measurement results from the interference of IGFBPs in the assay. Direct determinations in untreated serum samples (11) give false values because of the extremely slow dissociation of the IGF-I/IGFBP-3 complexes during the assay incubation. Depending on the ratio IGF-I to IGFBP in the sample interference comes up (see example Figure 1):

Figure 1. Interference of IGFBP in IGF-I measurements.



Known concentrations of IGF-I were assayed in the presence of 0.5 ng (left) or 5 ng (right) hIGFBP-3 by a conventional (□)and by the IGFBP-blocked assay (*).

Therefore, various techniques were applied to physically separate IGF-I from its binding proteins before measurement, including (a) size exclusion chromatography under acidic conditions, (b) solid-phase extraction and (c) acid-ethanol extraction (2,12,13). These techniques, however, are either inconvenient or time-consuming or give incomplete and not-reproducible recoveries. The most widely used method is the acid-ethanol extraction (13,14) with a recovery of only 70-80 % of IGFBP-bound IGF-I as a result of co-precipitation. The absolute results of such an extraction are therefore false low (15). The extraction removes the IGFBPs only insufficiently and leads to reduction in sensitivity of the assay due to pre-dilution of the samples by the extraction procedure. Furthermore, the remaining IGFBP may still interfere in the assay. In addition, the acid-ethanol extraction is ineffective in specimens other than serum or plasma (e.g. cell culture media), in which determination of IGF-I is already difficult enough due to the fact that IGFBPs are frequently present at large excess.

To avoid these difficulties, an uncomplicated assay was developed, in which special sample preparation is not required before measurement.

Reagents Provided

1)	MTP	**Microtiter plate**, ready for use: Microtiter plate with 96 wells, dived up in 12 strips of 8 wells (separately breakapart) coated with anti-mouse/rat IGF-I antibody.
2)	CAL	**Standards A-E**, lyophilised, contain recombinant IGF-I. Standard values are between **0.5 - 18 ng/ml** (0.5, 2.5, 6, 12 und 18 ng/ml) IGF-I and have to be reconstituted in **1 ml (each) Sample Buffer PP**. 50 µl per well are used in the assay. If the standards are required for more than one assay process we recommend to store the reconstituted Standards frozen at -20°C. **Attention:** Standards should be thawed only once – where required please store aliquoted in adequate volumes.
3)	DILU	**Sample buffer PP**, **125 ml**, ready for use, please use for the reconstitution of Standards A-E, Control Sera KS1 & KS2 and for the serum dilution.
4)	Control	**Control Sera KS1 & KS2**, each **500 µl** lyophilised: Sera have to be reconstituted in **500 µl Sample Buffer PP**. The IGF-I target values and the respective ranges are given on the vial labels. The dilution of the Control Sera KS 1&2 should be according to the dilution of the respective samples, the target values should be obtained by **multiplication** with the respective **dilution factor**.
5)	Ab	**Antibody Conjugate AK, 7 ml,** ready for use, contains the biotinylated anti-IGF-I antibody. Use 50 µl for each well in the assay.
6)	CONJ	**Enzyme Conjugate EK, 12 ml,** ready for use, contains horseradisch-peroxidase conjugate to streptavidin, use 100 µl for each well in the assay.
7)	WASHBUF 20x	**Washing Buffer (WP), 50 ml, 20 X concentrated** solution. **Washing Buffer (WP)** has to be diluted 1:20 with distilled or demineralised water before use (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill up with A.dest. to 1000 ml). Attention: After dilution the Washing Buffer is only 4 weeks stable, dilute only according to requirements.
8)	SUBST	**Substrate (S), 12 ml,** ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine.
9)	H_2SO_4	**Stopping Solution (SL), 12 ml,** ready for use, 0.2 M sulphuric acid, Caution acid!
10)		Sealing tape for covering of the microtiter plate, 2 x, adhesive.

Materials Required but not provided
Precision pipettes and multichannel pipettes with disposable plastic tips
Vortex-mixer
Microtiter plate shaker (350 rpm)
Microtiter plate washer (recommended)
Microplate reader ("ELISA-Reader") with filter for 450 and 620nm (or ≥590 nm)
Polyethylene PE/Polypropylene PP tubes for dilution of samples

WARNINGS AND PRECAUTIONS

For research use only. For professional use only.
Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.

Before use, all kit components should be brought **to room temperature at 20 - 25°C**. Precipitates in buffers should be dissolved before use by thorough mixing and warming.

Do not mix reagents of different lots. Do not use expired reagents.

The microplate contains snap-off strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.

Caution: This kit contains material of human and/or animal origin.

No known test methods can offer total assurance of the absence of infectious agents; therefore all components and specimens should be treated as potentially infectious.

Following components contain < 0.01% 2-Methyl-4-isothiazolin-3-one solution as preservative:**A-E, AK, EK, PP**

R34 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S36/37 Wear suitable protective clothing and gloves
S45 In case of accident or if you feel unwell seek medical advice

Following components contain < 0.01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-isothiazol-3-one as preservative: **A-E, AK, EK, PP, WP**

R36/38 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice S28.1 After contact with skin, wash immediately with plenty of water

Stop solution contains 0.2 M Sulfuric Acid (H_2SO_4)

R36/38 Irritating to eyes and skin
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water
S36/37 Wear suitable protective clothing and gloves.

Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step. Use separate pipette tips for each sample, control and reagent to avoid cross contamination. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.

TMB-Substrate (S) contains 3,3´,5,5´ Tetramethylbenzidine. Store and Incubate in the dark.

R20/21/R22	Harmful by inhalation, in contact with skin and if swallowed
R36/37/38	Irritating to eyes, respiratory system and skin
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1	After contact with skin, wash immediately with plenty of water
S36/37	Wear suitable protective clothing and gloves

General first aid procedures:
Skin contact: Wash affected area thoroughly with water. Discard contaminated cloths and shoes.
Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids.
Ingestion: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.
Do not eat, drink or smoke in these areas.
Never pipette the materials with the mouth.
Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.

PRINCIPLE
This ELISA for mouse/rat IGF-I is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The IGF-I in the sample binds to the immobilized first antibody on the microtiter plate, the biotinylated and Streptavidin-Peroxidase conjugated second specific anti-IGF-I-Antibody binds in turn to the immobilized IGF-I. In the closing substrate reaction the turn of the colour will be high specific catalysed, quantitatively depending on the IGF-I-level of the samples.

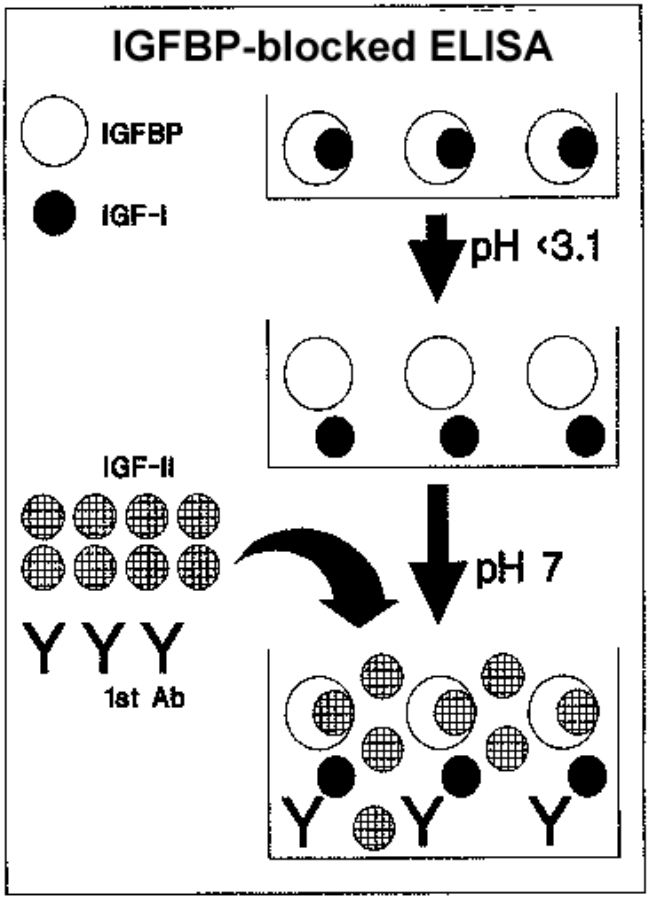


Figure 2: Principle of the IGFBP blocked IGF-I ELISA

In order to dissociate IGF-I from the IGFBPs, the samples must be diluted in an acidic buffer (Figure 2). The diluted samples are then pipetted into the wells, by this the pH-value will be neutralized. After neutralization of the samples, the excess IGF-II occupies the IGF-binding sites of the binding proteins, thus allowing the measurement of resulting free IGF-I. With this method, the IGFBPs are not removed, but their function and therefore their interference in the assay is neutralized. Due to the extremely low cross-reactivity of the IGF-I antibody with IGF-II, the excess of IGF-II does not disturb the interaction with IGF-I.

The test runs like a conventional ELISA using a Streptavidin-Peroxidase-Enzyme Conjugate.

Specimen

Serum samples as well as Heparin-, EDTA- and Citrat-Plasma samples are suited. Possible dilution of the sample by the anticoagulant must be considered.

Influence of Heparin (30IE/mL), EDTA (6,8mM) and NaCitrat (0,015M) on the measurement of IGF-I has been investigated in recovery experiments. Buffer solution was enriched with recombinant IGF-I and the above mentioned substances. No significant influence on the recovery of IGF-I was detected, on average the recovery of recombinant material in comparison to enriched PBS was 108%.

Cell culture medium is suitable as sample matrix after predilution of 1:2 with Sample Buffer PP.

Haemolytic reactions have to be avoided. The blood has to be allowed to clot and after complete clotting, serum is separated by centrifugation.

Storage of the samples

Storage at RT max. 2 days

Storage at –20°C max. 2 years

More than 2 freeze/thaw cycles are not recommended. IGF-I in samples was found to be unstable under repeated freeze/thaw cycles, measured values declined respectively.

Sample Preparation

Samples have to be diluted at least 1:10 in Sample Buffer (PP).

A serum dilution of 1:100 is in general suitable. However, the IGF-I levels can vary individually significant, we would therefore recommend to check this and adjust the dilution respectively.

After reconsititution, mix gently and incubate at room temperature for at least 15 minutes, but no longer than 2 hours.

Technical Notes

The assay has to be conducted strictly according the test protocol herein.

Reagents with different lot numbers cannot be mixed. The microtiterplate and reagents are stable until the indicated expiry if stored unopened and protected from sunlight at 2 – 8°C.

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming.

Incubation at room temperature means: 20-25°C

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtitre plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must become adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/or false values, excessive shaking may result in high optical densities and/or false values.

Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems.

All washing must be performed with the provided washing buffer diluted to usage concentration. Washing volume per washing cycle and well must be 300 µl at least.

The danger of handling with potentially infectious material must be taken into account.

When using an automatic microtitre plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non fuzzy absorbent tissue.

Manual washing is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamically swinging out the microtitre plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non fuzzy absorbent tissue.

Standards and Controls

For the reconstitution of the lyophilised components (Standards A - E and Control Sera KS1 &KS2) the kit Sample Buffer PP has to be used. It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam!) with a Vortex mixer.

The reconstituted standard and controls can be stored for 2 months at –20°C. Repeated freeze/thaw cycles have to be avoided.

Washing Buffer

The required volume of washing buffer is prepared by 1:20 dilution of the provided 20-fold concentrate with deionised water. The diluted Washing Buffer is stable for 4 weeks at 2-8°C. It has to be at room temperature for usage!

Microtiter plate

Unused microtiter plate stripes have to be stored airtight together with the desiccant bag at 2-8°C. The labelled expiry is not influenced in case of proper storage.

Assay Procedure
All determinations (Standards, Control Sera KS1 & KS2 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

When performing the assay, the Standards, Control Sera and the samples should be pipette as fast as possible (e.g., <15 minutes).

All incubations have to conducted at room temperature (20-25°C)

To avoid distortions due to differences in incubation times, Antibody (AK) and Enzyme Conjugate (EK) as well as the following Substrate Solution S should be added to the plate in the same order and in the same time interval as the samples. Stop Solution SL should be added to the plate in the same order as the Substrate Solution.

1) Add **50 µl Antibody Conjugate AK** in **all** wells used

2) Add **50 µl Sample Buffer PP** into the first two wells (These wells serve as blanks). Subsequently add 50 µl Standard or 50 µl of diluted Control Sera or diluted samples into wells designated for standards, controls or samples.

3) Cover the wells with sealing tape and incubate the plate for **1 hour** at **room temperature** (shake at 350 rpm)

4) After incubation, aspirate the contents of the wells and wash the wells **5 times 300 µl Washing Buffer WP / well.** The washing buffer should incubate for at least for 15 seconds/cycle. Tap the plate firmly against several layers of folded paper towels to remove excess washing buffer.

5) Following the last washing step pipette **100 µl** of the **Enzyme Conjugate EK** in each well.

6) Cover the wells with sealing tape and incubate the plate for **0.5 hour** at **room temperature** (shake 350 rpm).

7) After incubation wash the wells **5 times** with Washing Buffer as described in step 4

8) Pipette **100 µl of the TMB Substrate** Solution in each well.

9) Incubate the plate for **30 minutes in the dark at room temperature (20 - 25°C)**.

10) Stop the reaction by adding **100 µl of Stopping Solution**.

11) Measure the colour reaction within 30 minutes at 450nm (reference filter ≥590 nm).

Calculation of Results

Establishing the Standard Curve

For the evaluation of the assay it is preconditioned that the absorbance values of the blank should be below 0.25, these of standard E should be above 1.0.

Samples, which yield higher absorbance values than Standard E are beyond the standard curve, for reliable determinations these samples should be tested anew with a higher dilution.

Standards are provided in the following concentrations (use the concentration unit as preferred):

Standard	**A**	**B**	**C**	**D**	**E**
ng/ml	0.5	2.5	6	12	18

1) Calculate the **mean absorbance** value for the blank from the duplicated determination (well A1/A2).

2) Subtract the mean absorbance of the blank from the mean absorbances of all other values.

3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.

4) Recommendation: Calculation of the standard curve should be done by using a computer program. **A higher-grade polynomial,** or **four parametric logistic (4-PL) curve fit** or **non-linear regression** are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).

5) The IGF-I concentration of the diluted sample or the diluted control sera KS1&2 in ng/ml (or µg/ml according the chosen unit for the standards) is calculated in this way, the IGF-I concentration of the **undiluted sample** and of KS1 & KS2 is calculated **by multiplication** with the respective dilution factor.

The exemplary shown standard curve in Fig.3 **cannot be used** for calculation of your test results. You have to establish a standard curve for each test you conduct!

Exemplary calculation of the IGF-I concentration of a diluted sample:

Measured extinction of your sample 0.70
Measured extinction of the blank 0.02

Your **measurement programm** will calculate the IGF-I concentration of the diluted sample automatically by using the difference of sample and blank for the calculation. You only have to determine the most suitable curve fit (here: polynomial 3rd degree).

In this exemplary case the following equation is solved by the programm to calculate the IGF-I concentration in the sample:

$$y = -0.031924+0.2606x-0.010994x^2 +0.0019791x^3$$
$$3.0921= x$$

If the dilution factor (**1:100**) is taken into account the IGF-I concentration of the undiluted sample is 309.21 ng/mL

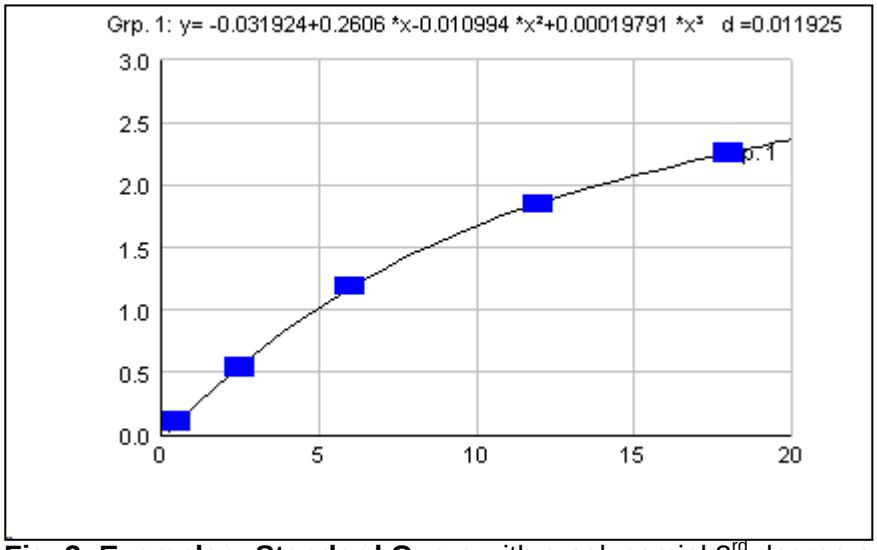


Fig. 3: Exemplary Standard Curve with a polynomial 3rd degree as curve fit.

Performance Characteristics

Standards

The Standards of this ELISA are prepared from **recombinant IGF-I** in concentrations of **0.5, 2.5, 6, 12, 18 ng/mL**.

Sensitivity

The analytical sensitivity of this ELISA yields < 0.029 **ng/mL**

Interassay Variability

	Sample 1	Sample 2	Sample 3
Mean	471	744	347
SD	24.81	41.8	23.3
VC%	**5.3**	**5.6**	**6.72**
n	10	10	16

Intraassay Variability

	Sample 1	Sample 2	Sample 3
Mean [µg/mL]	165	794	514
SD	8,0	47	22
VC%	**4,9**	**5,9**	**4,2**
n	10	10	10

Linearity

ng/mL	**Probe 1**	**Probe 2**
1:50	261	888
1:75	332	909
1:100	315	980
1:200	281	960
1:400	290	994
1:600	298	997
1:800	325	983

Method Comparison

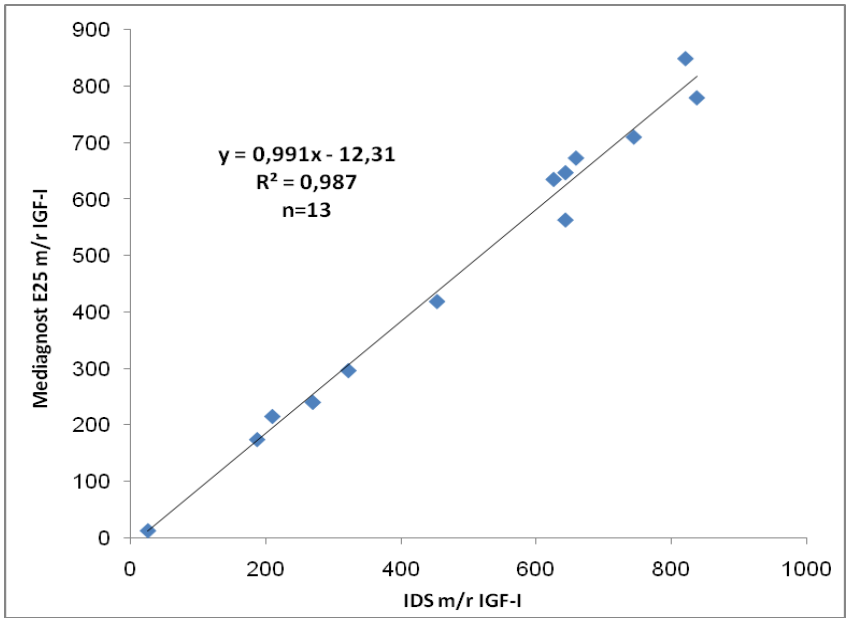


Figure 4: Method comparison of this IGF-I ELISA and another commercially available IGF-I ELISA.

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SUMMARY – IGF-I (MOUSE/RAT) ELISA 22-IG1MS-E01

Reconstitution / Dilution of Reagents		
Standards A-E	Reconstitution in **Sample Buffer PP**	**1 ml**
Control Serum KS1	Reconstitution in **Sample Buffer PP**	**500 µl**
Control Serum KS2	Reconstitution in **Sample Buffer PP**	**500 µl**
Wash Buffer WP	dilute in **A. dest.** (eg. total volume of 50 ml in a graduated flask and fill up to 1000 ml)	**1:20**
Sample + Control Sera KS1 and KS2: dilute 1:100 in Sample Buffer PP, mix immediately, incubate at least for 15 min, max. 2h. Use 50 µl for each well in the assay.		
Before conducting the assay equilibrate all **reagents to room temperature.**		

Assay Procedure for Double Determinations:

Pipette	**Reagent**	**Position**
50 µl	Antibody Conjugate **AK**	in **all** wells used
50 µl	Sample Buffer **PP** (blank)	A1 and A2
50 µl	Standard **A** (**0.5 ng/ml**)	B1 and B2
50 µl	Standard **B** (**2.5 ng/ml**)	C1 and C2
50 µl	Standard **C** (**6 ng/ml**)	D1 and D2
50 µl	Standard **D** (**12 ng/ml**)	E1 and E2
50 µl	Standard **E** (**18 ng/ml**)	F1 and F2
50 µl	Control Serum **KS1**	G1 and G2
50 µl	Control Serum **KS2**	H1 and H2
50 µl	Samples	following wells
Cover the wells with the sealing tape.		

Incubation: 1 h at **RT**, **350 rpm**

5x 300 µl	Aspirate the contents of the wells and wash **5x** with **300 µl Wash Buffer WP**	each well
100 µl	Enzyme Conjugate **EK**	each well

Incubation: 30 min at **RT**, **350 rpm**

5x 300 µl	Aspirate the contents of the wells and wash **5x** with **300 µl Wash Buffer WP**	each well
100 µl	Substrate **S**	each well

Incubation: 30min in the dark **RT**

100 µl	Stop Solution **SL**	each well
Measure the absorbance within **30 min** at **450 nm** with **≥ 590 nm** as reference wavelength.		